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Recovery and detection of *Cryptosporidium parvum* oocysts from water samples using continuous flow centrifugation

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Abstract

Continuous flow centrifugation (CFC) was used in conjunction with immunomagnetic separation (IMS) and immunofluorescence microscopy (IFA) and nested PCR to recover and detect oocysts of *Cryptosporidium parvum* and cysts of *Giardia intestinalis* from 10 L volumes of source water samples. Using a spiking dose of 100 oocysts, nine of 10 runs were positive by IFA, with a mean recovery of 4.4 ± 2.27 oocysts; when another 10 runs were analyzed using nested PCR to the TRAP C-1 and Cp41 genes, nine of 10 were positive with both PCR assays. When the spiking dose was reduced to 10 oocysts in 10 L, 10 of 12 runs were positive by IFA, with a mean oocyst recovery of 3.25 ± 3.25 oocysts. When 10 cysts of *Giardia intestinalis* were co-spiked with oocysts into 10 L of source water, five of seven runs were positive, with a mean cyst recovery of $x = 0.85 \pm 0.7$. When 10 oocysts (enumerated using a fluorescence activated cell sorter) were spiked into 10 L volumes of tap water, one of 10 runs was positive, with one oocyst detected. For the majority of the source water samples, turbidities of the source water samples ranged from 1.1 to 22 NTU, but exceeded 100 NTU for some samples collected when sediment was disturbed. The turbidities of pellets recovered using CFC and resuspended in 10 mL of water were very high (exceeding 500 NTU for the source water-derived pellets and 100 NTU for the tap water-derived pellets). While not as efficient as existing capsule-filtration based methods (i.e., US EPA methods 1622/1623), CFC and IMS may provide a more rapid and economical alternative for isolation of *C. parvum* oocysts from highly turbid water samples containing small quantities of oocysts.
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Keywords: Continuous flow centrifugation; *Cryptosporidium parvum*; Detection

1. Introduction

A variety of techniques have been evaluated for the recovery and detection of *Cryptosporidium parvum* oocysts from source and finished water samples [1]. The most widely used technique has been cartridge filtration in conjunction with immunomagnetic separation and immunofluorescence microscopy (i.e., US EPA method 1622/23). An extensive evaluation of the US EPA method 1622 for *C. parvum* detection was conducted by two laboratories. One laboratory obtained oocyst recoveries ranging from 20.7% to 61.2%, respectively, in reagent and source-quality water samples (10 L) spiked with approximately 100 oocysts [2]. The other laboratory obtained an average 49% recovery when 100 L volumes of tap water were spiked with 51 (four replicates) or 16 (six replicates) oocysts [3]. When molecular biology-based methods such as RT-PCR and cell culture were used as part of this protocol, one viable oocyst per 60 L of environmental water was detected [4]. However, the genus-specific character of these *C. parvum hsp70* primers has been questioned [5]. Using the US EPA method 1623, in conjunction with 18S rRNA gene nested PCR, Sturbaum et al. [6] reported successful

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detection of as few as five oocysts spiked into 1 L of source water.

Other recovery and detection methods that have been evaluated include membrane filtration and flocculation [1], membrane filter dissolution [7–9], and continuous flow centrifugation (CFC) [10]. When 45 L volumes of source water were spiked with 2.6×10^4 oocysts, the mean recovery for three replicates was 26.8%, compared to 0.5% for cartridge filtration and 18.7% for calcium carbonate flocculation [10]. Swales and Wright [11] also compared CFC with cartridge filtration, on 100 L water samples with nephelometric turbidity unit (NTU) values of 1–5. They spiked the water with 10 oocysts/L. Mean recoveries for the CFC method were 14.8% and 13.0% for the 1 and 5 NTU samples, respectively, compared to 9.7% and 9.0% for cartridge filtration, respectively. More recently, Borchardt and Spencer [12] used blood cell separator centrifuges and reported recovery efficiencies of 78% when 500 oocysts were spiked into 25 L of pond water, and 97% when 600 *Giardia* cysts were spiked into 30 L of pond water.

These results suggested that CFC offered recoveries equivalent to cartridge filtration, with improved economy and ease of use compared to filter-based methods. We report here on our evaluation of CFC for recovering *Cryptosporidium* oocysts, and *Giardia intestinalis* cysts, from spiked source water samples.

2. Materials and methods

2.1. Experimental design and collection of water samples

The term "run" is used to refer to the entire assay in which the spiked water sample is processed using CFC, subjected to overnight bead capture, and subsequent IFA microscopy the following morning.

The term "oocyst recovery" refers to the number of oocysts detected following a CFC run. The term "method sensitivity" refers to the percentage of CFC runs that were successful in detecting spiked oocysts.

The experimental design was as follows: in the first series of experiments, 10 source water samples (10 L) were spiked with 100 oocysts of *Cryptosporidium parvum* oocysts, subjected to CFC and IMS, and the pellet of recovered oocysts was enumerated using immunofluorescence antibody (IFA) staining and microscopy. In the second series of experiments, 10 source water samples (10 L) were spiked with 100 oocysts, subjected to CFC, and the recovered oocysts assayed by nested PCR for two *C. parvum* genes. These two series of experiments were performed in 2001. A third series of experiments, taking place in the Spring and Summer of 2002, was conducted on source water samples (10 L) spiked with 10 oocysts. Following CFC and IMS, the recoveries of oocysts and cysts were determined by immunofluorescence IFA microscopy. For seven of these 12 runs *Giardia* cysts were also spiked into the 10 L of source water; for these "dual spiked" samples, the first overnight IMS incubation was done using the Aureon anti-*Giardia* kit. The 10 mL, post-bead capture sample was then subjected to overnight incubation using the Dynal anti-*Cryptosporidium* kit, with 1 mL each of buffers A and B simply being added to the sample, along with the required 100 μL of anti-*Cryptosporidium* Dynalbeads.

A fourth series of experiments used 10 fluorescence-activated cell sorter (FACS)-enumerated oocysts as a spiking dose in 10 L of tap water. During Fall 2002, a total of 10 runs were done, with one unspiked control run done at the midway point. The oocyst pellets from this series of experiments were subjected to overnight IMS and IFA microscopy.

Source water samples were obtained from facilities at the Washington Suburban Sanitary Commission (WSSC) in Laurel, Maryland, and from Beaver Dam Creek (BDC) located on the campus of the Beltsville Agricultural Research Center at Beltsville, MD. Carboys (20 L) of water were stored at 4°C until used (later changed to room temperature; once the CFC protocol had been finalized it was routine to assay 60–80 L of water within a 1-week period). For experiments using tap water, 10 L volumes were obtained from taps located in building 173 of the Beltsville Agriculture Research Service campus.

2.2. Oocyst enumeration

Cryptosporidium parvum oocysts of the Beltsville-1 strain, as well as *Giardia intestinalis* cysts, were recovered from the feces of experimentally infected calves [13]. Briefly, the fecal material was sieved to remove larger particulates and then subjected to cesium chloride gradient centrifugation in a 250 mL centrifuge tube, at $250 \times g$ for 1 h at 5°C. The layer containing the oocysts (approximately 10–20 mL) was transferred to a 250 mL centrifuge tube, deionized water was added to bring the volume to 250 mL, and the tube was centrifuged at $250 \times g$ for 15 min at 15°C. The resultant pellet was washed twice with deionized water, and resuspended in a volume of 100–200 mL. Oocysts used for spiking were stored for no longer than 8 weeks after isolation.

Enumeration of oocysts used to create stock concentrations was accomplished by pipetting 10 μL of the oocyst suspension onto a Neubauer hemacytometer and counting them under bright field microscopy. A single count consisted of the mean of four different counts at the outer 4×4 grid of the hemacytometer. Counts from the hemacytometer method were used to prepare stock concentrations of oocysts at 1×10^6/mL.

For experiments in which oocysts were spiked into 10 L volumes of tap water, 10-oocyst aliquots in 10 μL of

sterile water (enumerated using a fluorescence activated cell sorter, or FACS) were purchased from the Wisconsin State Laboratory of Hygiene Environmental Health Division Flow Cytometry laboratory (Madison, WI).

To count oocysts recovered from continuous flow centrifugation experiments, as well as dilutions of oocysts used for source water spiking experiments, 1 μL of oocyst suspension was mixed with 1 μL of Merifluor™ immunofluorescence reagent (Meridian Diagnostics, Cincinnati, OH), pipetted onto each well of a 3-well Teflon-coated microscope slide (Cell-line™, Erie Scientific, Portsmouth, NH), and examined at 400× magnification (this emendation of the Merifluor protocol was done because in our experience, performing the stain washing step while oocysts are on the microscope slide can sometimes result in loss of oocysts and consequently lead to erroneous counts). The slides were examined via IFA microscopy by the same two individuals throughout the experiment to ensure consistency in oocyst identification and enumeration.

2.3. Continuous flow centrifugation/immunomagnetic separation

A photograph of the setup for the CFC is shown in Fig. 1A and a flow chart of the procedure is given in Fig. 1B. Oocysts were first spiked into 50 mL of sample water in a conical centrifuge tube, vortexed, and then deposited into the carboy containing the remaining 9.95 L of water. The carboy was shaken and then the contents pumped, using a MasterFLex peristaltic pump (Cole-Parmer, IL), through a No. 11 Model continuous flow centrifuge (Lavin, Hataboro, PA) at a rate of 200 mL/min, at a speed of 7140 rpm ($4200 \times g$). The Lavin CFC is 11.5 in (29.2 cm) high, 12 in (30.4 cm) in diameter, and weighs 35 lbs (15.8 kg). The body and lid are aluminum, and the rotor is stainless steel. The maximum rpm is 10,000 ($8550 \times g$). A diagram of the Lavin CFC operating principle and other pertinent information is available at: www.lavincentrifuge.com. After passage of the entire 10 L of oocyst spiked source water, 1 L of tap water was added to the carboy, shaken to clean the sides and top of the carboy, and pumped through the centrifuge. Pelleted material (approximately 130 mL) was collected from the bowl of the CFC and transferred to a 200 mL polypropylene conical centrifuge tube. The bowl was then washed with approximately 30 mL of water, which was added to the 200 mL conical tube; the combined eluate and wash volume of 160 mL was brought up to 200 mL with sterile water, and the tube was centrifuged in a swinging bucket rotor at 2800 rpm ($1500 \times g$) for 20 min. All but approximately 5 mL of supernatant was aspirated and discarded. The pellet was then resuspended in the 5 mL of supernatant and transferred to a 15 mL polypropylene conical tube; 10 mL of sterile water were used to rinse the 200 mL tube, and this rinse then added to the 15 mL tube, which was then centrifuged in the swinging bucket rotor at $1500 \times g$ for 20 min. Fourteen milliliter of supernatant was aspirated and discarded. For those samples undergoing IMS, the final recovery volume of approximately 1 mL was brought up to 10 mL with sterile water and subjected to IMS, using the Dynal Dynabeads® anti-*Cryptosporidium* kit (Lake Success, NY) and the Aureon anti-*Giardia* IMS kit (ImmTech, Inc. New Windsor, MD), according to the manufacturer's instructions, save that bead incubation was performed overnight rather than for 1 h.

Unspiked control runs—consisting of either 10 L tap water or 10 L of source water—were performed to monitor for false-positive results due to the recovery of oocysts and cysts retained in the CFC apparatus (refer to Tables 2 and 3).

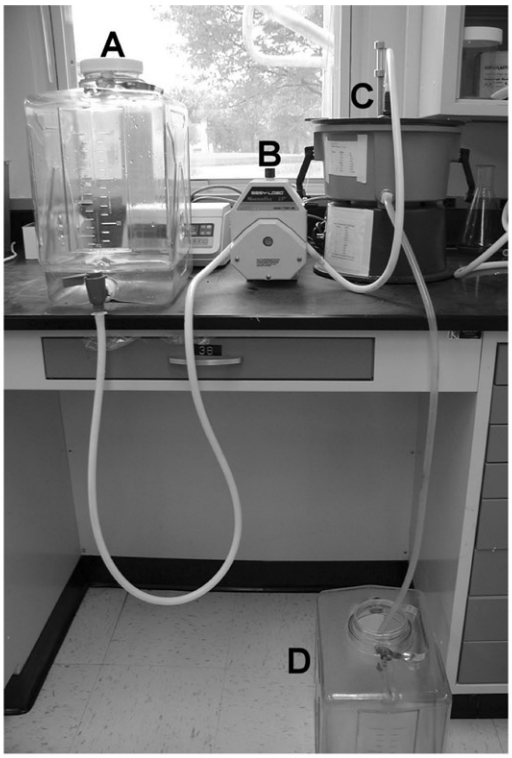
In between runs the CFC rotor was washed using a scrubbing brush and a soap solution and rinsed several times with tap water. The carboys used to deliver the spiked 10 L of source water were also cleaned with a soap solution and rinsed several times with tap water.

2.4. Instagene™ DNA extraction and PCR

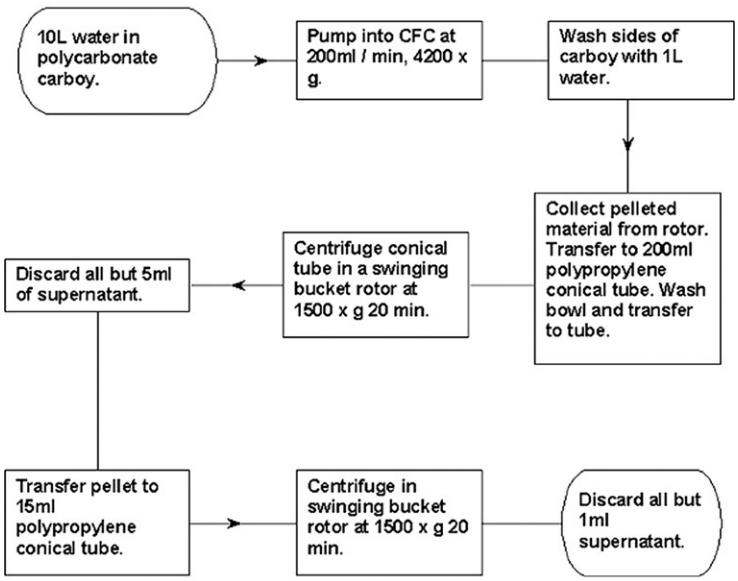
For the Dynal bead–oocyst pellets, a DNA extraction procedure using Instagene™ matrix (Bio-Rad, Hercules, CA) was performed. Briefly, the pellet (15–20 μL) in a 1.5 mL microfuge tube was subjected to two freeze–thaw cycles in a dry ice and methanol bath and 55°C water bath, then 100 μL of Instagene matrix was added and the tube incubated at 56°C for 15 min, followed by an incubation at 100°C for 8 min. The tube was then centrifuged to pellet the matrix and 10 μL of supernatant used as template for PCR.

For the Cp41 gene [14] Genbank Accession No. AF144621; the outer forward primer was Cp41OF: 5′ GAG GAG ATG GAC TAT TCT AGG; outer reverse primer Cp41OR: 5′ GCA ACA GTA GTA AGA GTG GTA; inner forward primer Cp41 IF: 5′ TGT ATG AAT TGG ATA TAT TAT TA; and inner reverse primer Cp41 IR: 5′ GTA AAA GCA ACA CCA TTA CTA. The TRAP C-1 primers Cp.Z, Cp.W, and Cp.E were used according to the protocol of Spano et al. [15] Cp.E and Cp.Z served as outer primers, and Cp.E and Cp.W as inner primers.

PCR was done in 50 μL volumes, containing 1 U *Taq* polymerase (Life Technologies, Gaithersburg, MD), 200 μM each dNTP, 1.5 mM $MgCl_2$, 5 μL 10× PCR buffer, and 50 pmol of each primer. Cycling parameters for the Cp41 gene PCR were: 95°C for 1 min, followed by 35 cycles of 95°C for 15 s, 50°C for 30 s, and 60°C for 1 min. Cycling parameters for the TRAP C-1 nested PCR followed the published protocol (Spano et al.,



(A)



(B)

Fig. 1. Panel A: continuous flow centrifugation (CFC) apparatus. A 10 L volume of source water in a plastic carboy (A) is pumped via a peristaltic pump (B) into a Lavin CFC (C) at a rate of 200 mL/min. Overflow from the rotor is discarded in another carboy (D). Panel B: flowchart depicting the steps in the CFC procedure.

1998), albeit with the PCR extension step time increased to 2 min.

3. Results

3.1. Oocyst enumeration

IFA microscopy examination of oocyst preparations used in spiking experiments showed that the observed counts were not markedly different than expected counts (i.e., the coefficient of variation did not exceed 10%). An example of one series of counts is provided in Table 1. Here, each replicate consists of 10, 1 μL aliquots with an expected content of 10 oocysts/aliquot. The average count of oocysts per sum of 10, 1 μL aliquots was 97.3 ± 8.0, with a range of 85–111 (CV = 8.0%). ANOVA performed on the data in this table indicated no significant difference in oocyst counts between replicates ($F = 0.6271 < F_{0.05\ 9,90} 1.99$; $P = 0.771$). We interpreted these results to indicate that our spiking doses did not contain significantly more oocysts than predicted from enumeration data.

3.2. Optimization of CFC parameters

A series of experiments was conducted using a spiking dose of 10,000 oocysts in 10 L of tap water. Percent recovery for 200 mL/min at $2800 \times g$ was 7%, for 200 mL/min at $4200 \times g$, 19%; for 250 mL/min at $4200 \times g$, 5%; for 200 mL/min at $4200 \times g$, 27.3%; and for 200 mL/min at $6000 \times g$, 20%. Consequently, CFC runs used a flow rate of 200 mL/min at $4200 \times g$ (note that on the Lavin CFC this is equivalent to a dial setting of "6").

3.3. Recovery of oocysts from spiked water samples using CFC

In general, it took 2.5 h to perform the CFC procedure, and another 12 (i.e., overnight) to conduct the IMS separation. Subsequent IFA microscopy on purified oocysts usually took under 1 h. DNA extraction using Instagene matrix, followed by nested PCR, added another 4 h to the 14.5 h devoted to CFC and IMS.

CFC pellets derived from source water were essentially sediment and ranged in size from 0.5 to 1.0 mL. NTU values for WSSC source water samples ranged from 1.1 to 3.9, except for one source water sample that had a value of 22. Readings for BDC source water were higher and ranged from 6.1 to 27.0 NTU (however, a collection taken on February 13, 2002 was particularly turbid due to inadvertent stirring up of sediment and readings of 134, 135, and 133 NTU were recorded). We observed very high NTU readings (> 500) on the pellet of sediment recovered by the CFC, even when mixed with 10 mL of water and 1 mL each Dynal kit buffers A and B for the initial IMS step; for example, of six samples of WSSC-derived source water assayed in June, 2001, the average NTU reading for the resuspended pellets was 982 (± 37.7, range 916–1014).

Preliminary experiments, consisting of three assays in which 1×10^3 oocysts were spiked into 10 L of WSSC source water, showed recoveries of 174, 115, and 135 oocysts (17%, 11%, and 13.5%, respectively), as determined by IFA microscopy. The success of the CFC technique in detecting these spiking doses led us to attempt recovery of smaller numbers of oocysts.

Accordingly, 10 runs were performed in which 100 oocysts were spiked into 10 L volumes of source water from the WSSC; oocysts were recovered using CFC and an overnight Dynal bead IMS step; and IFA microscopy was used to detect recovered oocysts. Another 10 runs

Table 1
Enumeration of *Cryptosporidium parvum* oocysts used in source water spiking experiments

Rep#	Well#										Total
	1	2	3	4	5	6	7	8	9	10	
1	11	16	9	11	2	5	10	8	9	16	97
2	5	9	6	12	8	4	8	13	9	13	87
3	11	10	9	9	9	9	4	13	15	9	98
4	10	10	7	5	11	9	8	11	15	18	104
5	13	11	10	8	8	8	10	14	7	8	97
6	8	14	10	11	15	14	9	8	10	12	111
7	13	10	15	10	9	10	8	7	9	8	99
8	9	14	11	2	9	8	9	11	9	3	85
9	8	8	9	6	13	9	16	11	12	12	104
10	11	10	14	6	3	13	7	7	10	10	91
											$x = 97.3 \pm 8.01$

Ten, 1 μL aliquots of oocysts were examined using MeriFluor IFA staining with 400× magnification.

were subjected to CFC and overnight IMS and examined by nested PCR for the *Cryptosporidium parvum* Cp41 gene, and the TRAP C-1 gene.

As shown in Table 2, of the 10 runs using IFA microscopy, nine successfully detected oocysts, although recoveries were low (average of 4.4 oocysts per assay).

Table 2
Recoveries of *Cryptosporidium parvum* oocysts spiked into 10 L volumes of source water and isolated using continuous flow centrifugation and immunomagnetic separation

Date	No. oocysts spiked	No. oocysts recovered[a]
Unspiked control	0	0
5.18.01	100	8
5.29.01	100	5
5.30.01	100	6
6.5.01	100	0
6.18.01	100	4
6.19.01	100	3
6.20.01	100	4
6.25.01	100	7
6.28.01	100	3
7.18.01	100	4
		$x = 4.4 \pm 2.27$

[a] Recovery determined by IFA microscopy of Dynal™ bead/oocyst pellet. Summary statistics: $x = 4.4 \pm 2.27$; range 3–8 oocysts; method sensitivity 90% (9/10).

Nine of the 10 runs subjected to DNA extraction with Instagene matrix, and nested TRAP C-1 gene and nested Cp41 gene PCRs, successfully amplified (Fig. 2). Oocysts were not observed in one unspiked control run subjected to IFA microscopy, and PCR assays on three unspiked run samples were also negative.

A third set of experiments (12 runs) used a spiking dose of 10 oocysts into 10 L of source water from BDC. As shown in Table 3, the mean oocyst recovery was 3.25, with a method sensitivity of 83% (10 of 12 assays). One sample (October 4, 2001) had a recovery of 12 oocysts, which is higher than the spiking dose of 10 oocysts; it is unclear if this represents inflation of the recovery due to the presence of naturally occurring oocysts in the source water sample, or is simply a consequence of statistical variability in the quantity of oocysts calculated to be in the spiking dose. One oocyst was detected in one of two source water unspiked controls, which we interpret to represent the presence of naturally occurring *C. parvum*. Both unspiked tap water control runs were negative (Table 3). In an effort to differentiate between spiked and naturally occurring oocysts, we conducted an additional six CFC runs in February and March 2002 in which 10 oocysts, prestained with Merifluor reagent, were spiked into 10 L of BDC water. However, no oocysts were recovered from these assays.

Because the recovery rates of oocysts in the experiments using a spiking dose of 100 oocysts were low, we

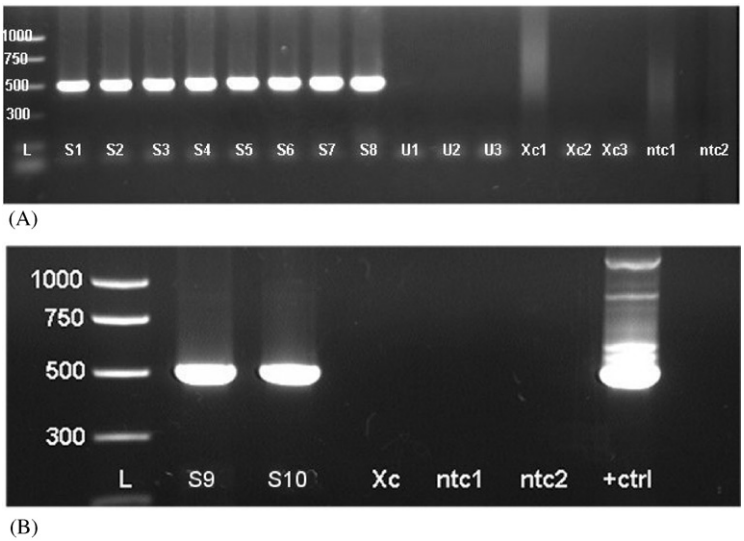


Fig. 2. Results of nested PCR performed on ten samples of DNA extracted from *Cryptosporidium parvum* oocysts recovered using continuous flow centrifugation (CFC). The TRAP C-1 nested primer set was used according to the protocol of Spano et al. [15]. Results from eight runs are shown in Panel A and from another two runs in Panel B. Panel A: Lane L: DNA ladder, with rung sizes indicated. Lanes S1–S8: recovered oocyst samples from spiked CFC runs. Lanes U1–U3: unspiked controls. Lanes Xc1–Xc3: DNA extraction controls. Lane ntc1: primary PCR no template control. Lane ntc2: secondary PCR no template control. Panel B: Lane L: DNA ladder, with rung sizes indicated. Lanes S9–S10: recovered oocyst samples from spiked CFC runs. Lane Xc: DNA extraction control. Lane ntc1: primary PCR no template control. Lane ntc2: secondary PCR no template control. Lane + ctrl: *C. parvum* positive control.

Table 3
Recoveries of *C. parvum* oocysts from 10 L volumes of source water spiked with 10 oocysts

Date	No. oocysts spiked	No. recovered[a]	Remarks
10.3.01	10	3	WSSC source water
10.4.01	10	12	
1.30.01	10	6	BDC source water
1.31.02	10	3	
2.1.02	10	1	
2.12.02	10	3	
2.14.02	10	4	
2.15.02	10	3	BDC source water
3.7.02	10	3	BDC source water
3.22.02	10	1	
3.26.02	10	0	
3.28.02	10	0	
10.10.02	0	0	Unspiked tap water control
10.18.02	0	0	Unspiked tap water control
2.5.02	0	1	Unspiked BDC source water
4.02.02	0	0	Unspiked BDC source water

[a] Recovery determined by IFA microscopy of Dynal™ bead/oocyst pellet. Summary statistics: $x = 3.25 \pm 3.25$; range 1–12 oocysts; method sensitivity 83% (10/12). WSSC: Washington Suburban Sanitary Commission (Burtonsville, MD). BDC: Beaver Dam Creek (Beltsville, MD).

investigated whether the bead capture step was responsible for reduced recovery. A set of three experiments was conducted in which 10 L of unspiked WSSC source water was subjected to CFC and the resultant pellet of sediment (0.5–1.0 mL) was mixed with 10 mL of water before IMS with the Dynal kit. This 10 mL volume was spiked with 1×10^3 oocysts and incubated overnight, followed by separation of the oocyst–bead pellet and examination with IFA microscopy. Numbers of recovered oocysts were 153, 81, and 151 (15.3%, 8.1%, and 15.1%, respectively) indicating that substantial numbers of oocysts were not being captured by the beads.

For seven of the 12 CFC runs described in Table 3, 10 cysts of *Giardia intestinalis*, prestained with Merifluor reagent, were co-spiked along with oocysts into 10 L of BDC water and recovered using the Aureon *Giardia* IMS kit (conducted from February 12 to March 28, 2002). Five of the seven *Giardia* "co-spiked" runs were positive (method sensitivity = 71%), with 1, 1, 1, 1, and 2 cysts recovered ($x = 0.85 \pm 0.7$). No cysts were detected in two unspiked BDC source water and two unspiked tap water controls.

A fourth series of experiments was conducted (in the Fall of 2002) using a spiking dose of 10 oocysts, enumerated using a FACS instrument, into 10 L of tap water from our laboratory located on the Beltsville campus. The CFC pellet was subjected to an overnight IMS step followed by IFA microscopy. Out of a total of 10 runs, only 1 was positive, for one oocyst. This gives a mean oocyst recovery of 0.1 and a method sensitivity of 10%. An unspiked control run done after the first five spiked runs was negative. Because of the aged condition of the pipes in our laboratory building, rust particles were visible in the pellets derived from the tap water CFC runs; the turbidities of these pellets when resuspended in 10 mL of sterile water with 1 mL each of Dynal buffers A and B were high, averaging 107.7 NTU ($n = 3$).

4. Discussion

We have evaluated a tabletop, portable continuous flow centrifuge, in conjunction with overnight IMS, for the isolation of *Cryptosporidium parvum* oocysts from spiked source water samples. When the spiking dose was 100 oocysts in 10 L, nine of 10 runs were positive by IFA microscopy, with a mean recovery of 4.4 oocysts per run. Another nine of 10 runs were positive by both nested PCR assays. When the spiking dose was reduced to 10 oocysts in 10 L, 10 of 12 runs were positive by IFA microscopy, with a mean recovery of 3.25 oocysts per run. When 10 cysts of *Giardia intestinalis* were spiked into 10 L of source water, five of seven runs were positive by IFA microscopy, with a mean recovery of 0.85 cysts per run. When tap water was assayed, using oocysts enumerated by a more accurate method (FACS) than IFA microscopy counts of diluted oocysts, only one of 10 runs was positive, for one oocyst. It should be noted that IFA microscopy on two aliquots of the FACS-enumerated oocysts (10 oocysts in 10 μL sterile water) resulted in counts of seven and four oocysts, indicating that almost 50% of the oocysts may be lost during the pipetting required to transfer the oocysts from their original 0.5 mL tube to the 0.5 mL tube containing Merifluor stain, and from there to the microscope slide used for IFA enumeration. If a similar loss of oocysts to pipetting steps applies to the CFC experiments, than the actual quantities of FACS-derived oocysts being spiked into the 10 L of tap water may be substantially less than 10. Another explanation for the poorer oocyst recoveries, observed in the spiked tap water samples, may be that the presence of particulate matter in the source water samples serves as a flocculating agent and thereby aids in oocyst concentration in the final pellet. Future experiments will investigate the use of diatomaceous earth as a flocculating agent for oocysts spiked into tap water.

For the 100 oocyst and 10 cyst spiking doses, in either source or tap water, our mean oocyst recovery and

method sensitivity was smaller than that reported by Zuckerman et al. [10], although these authors used much larger numbers of organisms in their experiments. For example, they spiked 45 L of source water with the equivalent of 577 oocysts/L, and achieved an average recovery of 26.8% of the spiking dose. When *Giardia* cysts (1.1×10^4) were spiked into 45 L of source water, these authors observed an average recovery of 56.7% of the spiking dose, compared to 8.2% with cartridge filtration and 43.4% with calcium carbonate flocculation. In comparison, Swales and Wright [11] spiked 100 L volumes of low turbidity (i.e., 1 NTU) water with 10 oocysts/L and reported an average recovery of 14.8% of the spiking dose, compared to 13% in higher turbidity water (5 NTU). We speculate that our reduced recoveries were influenced by the large quantities of silt and particulates in our CFC pellets, which probably influenced the efficacy of the antibody-binding portion of the IMS step [16]. It is also possible that after the initial CFC step, the subsequent two centrifugation steps, and associated liquid handling procedures, are other opportunities for loss of oocysts from the sample. Future experiments on the CFC method will attempt to eliminate one of these secondary centrifugations in the hopes of improving the recovery of oocysts.

More recently, Borchardt and Spencer [12] used blood cell separator centrifuges to perform continuous separation channel centrifugation (CSCC) on different water matrices spiked with varying quantities of *C. parvum* oocysts and *G. lamblia* cysts. After concentration, the recovered pellet containing oocysts and cysts was filtered through a 25 mm diameter, 1 μm sized polycarbonate membrane, which was stained with MeriFluor reagent and examined with fluorescence microscopy (200×). When pond water (25 L) was spiked with 500 oocysts, mean recovery was 78% ($n = 3$); when 30 L pond water was spiked with 600 cysts, mean recovery was 97% ($n = 3$). In contrast to our CFC protocol, the CSCC procedure did not require an IMS step, and could be accomplished (depending on the flow rate) within several hours. However, the authors noted that the blood cell separator centrifuges were expensive, and may be vulnerable to clogging and premature run termination when highly turbid (>100 NTU) water is subjected to analysis.

The CFC and IMS procedures did not appear to affect oocyst integrity or viability, in contrast to the filter dissolution method [9], but it was found that nested PCR was necessary to detect *C. parvum* DNA in the oocyst–bead pellet recovered from the samples receiving a 100-oocyst spike. A similar requirement for nested PCR, used in conjunction with traditional capsule filtration, to detect small numbers of oocysts spiked into water samples was observed by Sturbaum et al. [17,6] and Jellison et al. [18]. In contrast, a cell culture RT-PCR assay, using the *C. parvum* hsp70 gene as a target, successfully detected 1–10 oocysts in concentrates from 65 to 100 L of source water samples [4]. However, Kaucner and Stinear [5] reported that the hsp70 primer pair will amplify non-*Cryptosporidium* organisms present in source water samples, and we have observed amplicons approximately equal in size to those of the predicted size of the hsp70 product from BDC water samples that were negative by TRAP C-1 PCR and IFA microscopy (unpublished data). Hallier-Soulier and Guillot [19] reported detection of as few as one oocyst spiked into 20 L of source water when using capsule filtration in conjunction with IMS and one round of 18S rRNA PCR. However, given that Sturbaum et al. [6] reported nonspecific amplification of the dinoflagellate *Gymnodinium* from source water samples with their 18S rRNA primers, ancillary methods, such as sequencing or RFLP analysis of amplicons, may be necessary to validate PCR results achieved with the use of primers targeting this gene.

The approximate cost for the CFC apparatus we used is $9000 ($7039.00 for the centrifuge and $2000 for the peristaltic pump). This is a substantial financial outlay, but in contrast to capsule filters used in the US EPA method 1622/1623 such as the Envirochek from Pall Gelman Laboratories, Ann Arbor, MI, which cost ~$98 each [20], the inexpensive, plastic 200 and 15 mL centrifuge tubes are the only disposables associated with its use.

The oocyst recoveries we observed with the CFC method were inferior to those achieved with capsule filtration methods. To cite three very recent publications, LeChevallier et al. [21] reported that when 10 L source water was spiked with 500 oocysts and subjected to US EPA method 1623, recoveries from $n = 29$ samples averaged 72% (range 5.9–106.9%) (water turbidities were not provided). McCuin and Clancy [22] reported oocyst recoveries, from 50 L raw water samples spiked with ~100 oocysts, ranging from 19.5% to 54.5% (the protocol they used called for 10 L to be spiked and filtered, after which another 40 L were passed through the filter to give a total filtered volume of 50 L). Interestingly, DiGiorgio et al. [23] found that highly turbid water (NTU readings ranging from 11 to 99) allowed "High Volume" (HV) and "standard" Envirochek filters to accommodate a mean of 3.2 and 1.7 L of water, respectively, due to clogging with sediments. Recoveries from two sites (with three replicates per site) in which 10 L samples were spiked with ~100 oocysts, ranged from 0.47% to 53% with the HV filter and 0.83% to 61% with the standard filter. Accordingly, we believe that the CFC technique may offer some advantages in terms of recovery efficiency, cost, and ease of use, when highly turbid samples containing small quantities of oocysts (i.e., $<10\,L^{-1}$) require analysis.

5. Summary

1. Oocysts of *Cryptosporidium parvum* were spiked into 10 L volumes of source water and recovered using a portable continuous flow centrifuge (CFC) and overnight immunomagnetic separation (IMS). Recovered oocysts were detected using either IFA microscopy, or nested PCR for the Cp41 and TRAP C-1 genes.
2. When 10 L volumes of source water samples were spiked with 100 oocysts, nine of 10 runs were positive using IFA microscopy, with mean recovery of 4.4 oocysts per run. Nine of another 10 runs were positive by both Cp41 and TRAP C-1 nested PCR assays.
3. When 10 L volumes of source water samples were spiked with 10 oocysts, 10 of 12 runs were positive, with a mean oocyst recovery of 3.2 per run. One of two unspiked controls contained one oocyst; two unspiked tap water controls were negative.
4. When 10 prestained *Giardia intestinalis* cysts were co-spiked into 10 L volumes of source water, five of seven runs were positive, with a mean cyst recovery of 0.8 per run.
5. When 10 oocysts (enumerated via FACS) were spiked into 10 L volumes of tap water, only one of 10 runs was positive, with one oocyst recovered.
6. The entire procedure, from CFC to oocyst detection with IFA microscopy, took less than 24 h.

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